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$A^{-2} \rightarrow G$ Transition at the 3′ Acceptor Splice Site of IVS17 Characterizes the COL2A1 Gene Mutation in the Original Stickler Syndrome Kindred

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Hereditary progressive arthro-ophthalmopathy, or "Stickler syndrome," is an autosomal dominant osteochondrodysplasia characterized by a variety of ocular and skeletal anomalies which frequently lead to retinal detachment and precocious osteoarthritis. A variety of mutations in the COL2A1 gene have been identified in "Stickler" families; in most cases studied thus far, the consequence of mutation is the premature generation of a stop codon. We report here the characterization of a COL2A1 gene mutation in the original kindred described by Stickler et al. [1965].

Conformational sensitive gel electrophoresis (CSGE) [Ganguly et al., 1993] was used to screen for mutations in the entire COL2A1 gene in an affected member from the kindred. A prominent heteroduplex species was noted in the polymerase chain reaction (PCR) product from a region of the gene including exons 17 to 20. Direct sequencing of PCR-amplified genomic DNA resulted in the identification of a base substitution at the A^{-2} position of the 3′ splice acceptor site of IVS17. Sequencing of DNA from affected and unaffected family members confirmed that the mutation segregated with the disease phenotype. Reverse transcriptase-PCR analysis of poly A+ RNA demonstrated that the mutant allele utilized a cryptic splice site in exon 18 of the gene, eliminating 16 bp at the start of exon 18. This frameshift eventually results in a premature termination codon. These findings are the first report of a splice site mutation in classical Stickler syndrome and they provide a satisfying historical context in which to view COL2A1 mutations in this dysplasia.

KEY WORDS: Stickler syndrome, COL2A1 mutation, splice site mutation

INTRODUCTION

Hereditary progressive arthro-ophthalmopathy, more commonly known as Stickler syndrome, was first described by Stickler et al. [1965]. In this report, Dr. Stickler carefully reconstructed the clinical findings of a large Minnesota kindred which had been examined at the Mayo Clinic as early as 1897 by Dr. C.H. Mayo. The disease was characterized by autosomal dominant transmission; however, it was noted that the disease displayed "variable expressivity" with some degree of nonpenetrance in the original kindred. The primary ophthalmologic abnormality was a high degree of myopia and associated chorioretinal degenerative changes; the myopia was congenital and progressed during the first two decades in some individuals. There was also a secondary anomaly of total retinal detachment during the first decade of life without evidence of trauma. A third manifestation was the late appearance of complicated cataracts, keratopathies, chronic uveitis, or secondary glaucoma in myopic eyes that had undergone retinal detachment. Among the joint manifestations, there was abnormal development of articular surfaces with concomitant premature degenerative changes of joints and, in some members, mild hypermobility. The joints which were primarily affected included ankles, wrists, elbows, shoulders, and hips (metacarpophalangeal joints were involved infrequently). The articular symptoms began in childhood. There was clear radiographic evidence of unusual irregularity of articular surfaces suggestive of abnormal epiphyseal development. The irregular articular surfaces were apparent even before the loss of cartilage tissue. In members with hip involvement, subluxation of the hips was frequent. Stickler suggested in his initial communication that "... A biochemical defect involving connective tissue

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is postulated as a cause of hereditary progressive arthroophthalmopathy." True to this prediction, a variety of mutations in the type II procollagen (COL2A1) gene have been identified in some classical Stickler syndrome families; in most cases studied thus far, the consequence of COL2A1 mutation is the premature generation of a stop codon [Ahmad et al., 1991, 1993; Brown et al., 1992, 1993; Ritvaniemi et al., 1993]. However, the original kindred described by Stickler et al. [1965] had not yet been studied. We report here the characterization of a COL2A1 gene mutation in this family.

MATERIALS AND METHODS

Patients and Control Samples

Patient III-4, the index patient for this study, was a 66-year-old woman who had large knee and ankle joints since birth. This was diagnosed with chronic uveitis as a manifestation of the Stickler syndrome in 1960 at the age of 31 years. She lost vision in her left eye when she was struck by an acorn at the age of 6 years. Ophthalmologic examination showed a large retinal detachment of the nasal and inferior portion of the left eye. The right eye showed a high degree of myopia and, at age 11, detachment of the retina of the right eye occurred. During childhood and adolescence, she had pain in her knees and, less frequently, in her ankles, which often became erythematous and swollen with overuse. As a young adult, she practiced the piano daily for many hours and sometimes her wrists also became painful. As an adult, she had light perception in the right eye, and the left eye was blind and showed marked phthisis. She had removal of the left eye because of pain and for cosmetic reasons in 1965 with placement of an implant. She worked as a piano teacher. She continued to show enlargement of multiple joints including ankles and knees, when last examined at the Mayo Clinic at the age of 66 years. She had increasing pain and degenerative arthritis in her hips and knees, requiring increased pain medication; however, she had not required any joint replacement surgery.

The index patient's brother, member III-5, was a 64-year-old man. As originally reported by Stickler et al. [1965], the patient had mild myopia but, upon formal examination, no ophthalmologic findings of Stickler syndrome were observed. His joints were clinically and radiographically normal. At age 50, radiographs of the lumbar spine and hips were obtained as part of a follow-up study of the family; these were normal. He was evaluated by the Mayo Clinic ophthalmologist most recently in 1985 at the age of 54 years and at that time he still had no ophthalmologic signs of Stickler syndrome. He did not submit to reexamination at the time of the current study, but he provided a blood specimen and reported that he remained without joint disease. He had 3 healthy children, one of whom, IV-7, is described below. He had two healthy grandchildren. He was considered unaffected.

The index patient's brother, member III-10, was a 52-year-old man with Stickler syndrome. He was reported to have had large knee and ankle joints at birth. He was born with moderately severe bilateral talipes calcaneovarus which was treated with casts, splints, and special shoes. At the age of 2 years, he was thought to have some beading of the costochondral junction and contraction of the ribs anteriorly. He was entered into a school for the visually impaired at age 9 years, at which time his right eye showed a high degree of myopia and astigmatism with acuity of 20/100. The left eye had a dense cataract with acuity limited to light perception. When he was examined in 1962 at age 19, in addition to the high degree of myopia in the right eye, a vitreous band was seen. At age 13, he began to experience pain in his knees after exercise. At that time, physical examination showed enlargement of the elbows and wrists with marked valgus deformity in both knees. When he was 16, he underwent triple arthrodesis of both ankles and an osteotomy of the left knee. He had limited glenohumeral abduction of the shoulder joints and his patellae were dislocated laterally. Radiographic evaluation documented narrowing of the articular spaces of the knee joints with minimal irregularity of the articular surfaces as seen in degenerative joint disease. We did not have the opportunity to reexamine this individual; however, he was willing to provide a blood specimen for the current study. He indicated that he was "going blind" with tunnel vision in both eyes. He had engaged in his family trade of piano tuning.

Member IV-7, the niece of the index patient, was born after the original report by Stickler et al. [1965]. She presented for evaluation in 1994 because of "floaters" in her eyes that her optometrist thought might be related to her family history of Stickler syndrome. She underwent complete physical examination and was a healthy appearing young woman with no clinical signs of Stickler syndrome. She also underwent a complete ophthalmologic examination by the ophthalmologist who had participated in monitoring of other members of her family. He saw no ophthalmologic evidence of Stickler syndrome. She also had X-rays of the pelvis and hips with no radiographic signs of joint disease. She was considered unaffected.

The index patient's sister, member III-3, who had known Stickler syndrome, died of renal failure due to Goodpasture disease at age 75 years.

Although no new relatives have been diagnosed with Stickler syndrome in the portion of the family with which we had contact since the original report, it is of note that our index patient's two affected sibs elected not to have children. Of our index patient's 4 children, only the eldest was affected and he too, as of this time, has elected not to have children.

Genomic DNA from relatives and unrelated controls was isolated from 10 ml of whole blood on an automated DNA extractor using conditions specified by the manufacturer (GenePure; Applied Biosystems Inc., Foster City, CA). DNA samples were stored at −20°C. Control samples were chosen from a random population of disease-free donors. Control samples had been previously characterized for sequence variations in the COL2A1 gene by direct sequencing of PCR-amplified genomic DNA [Williams et al., 1992].

CSGE Analyses of PCR-Amplified Genomic DNA

The PCR products from the COL2A1 gene ranged in size from 291 bp to 811 bp and included all 54 exons of

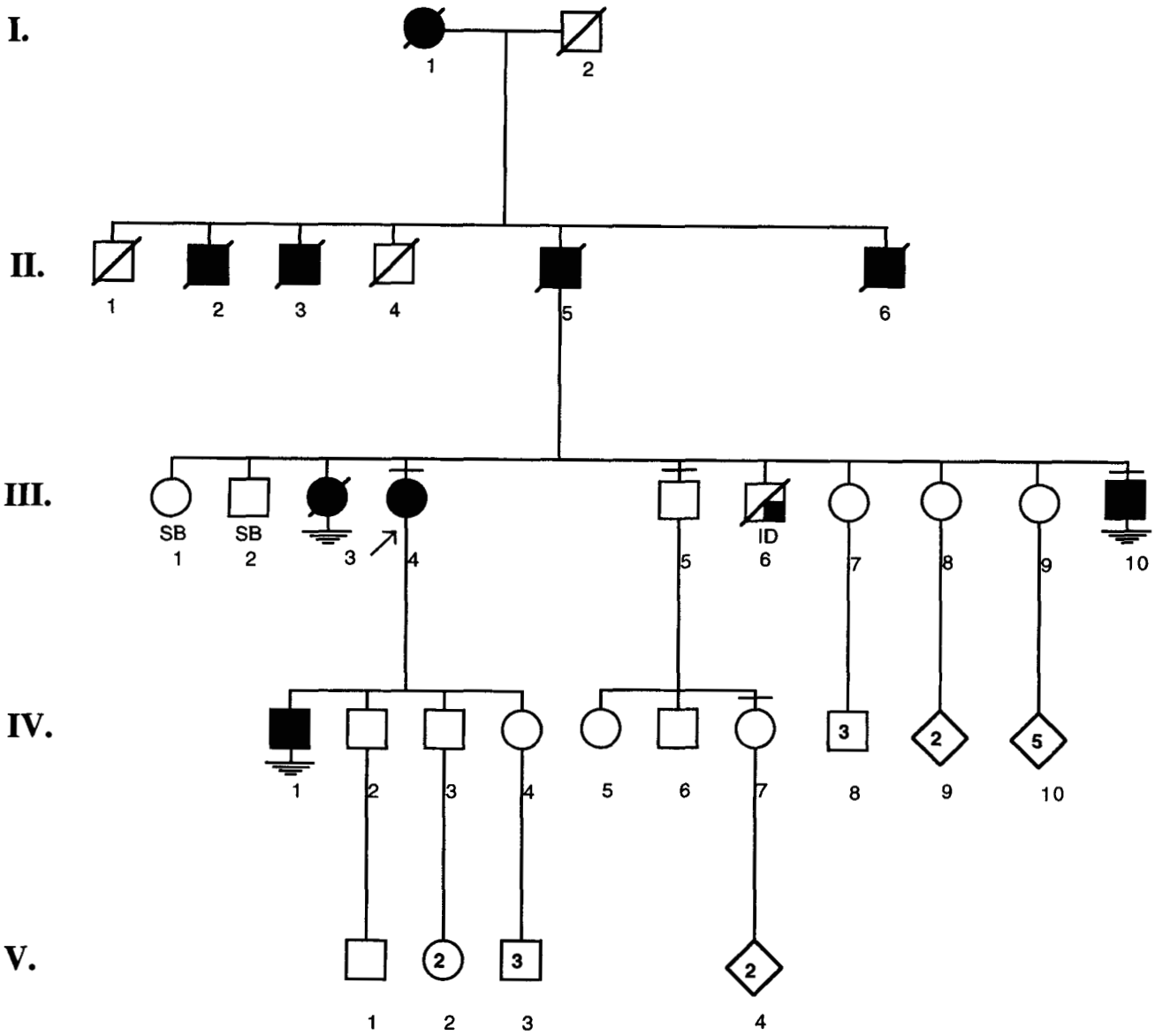

Fig. 1. Pedigree of the family originally reported by Stickler et al. [1965]. In the original pedigree, member III-4 was IV-9, member III-5 was IV-10, and member III-10 was IV-15. Patient IV-7 was born after the original pedigree was reported. Member III-6 died at 4 days of age (ID) and had a cleft palate. Index case is member III-4. Members with bars above their respective symbols indicate those individuals included in the current study. Members with multiple bars below their respective symbols indicate individuals who have elected not to have offspring. SB: stillbirth. Members III-4 and III-10 were found to harbor the splice site mutation described in Figure 2. Members III-5 and IV-7, who were clinically unaffected, did not carry the mutation.

the gene plus all intron/exon boundary regions. The primers for amplification of the COL2A1 gene have been described elsewhere [Williams et al., 1992]. Prior to implementing the conformational sensitive gel electrophoresis (CSGE) procedure, the PCR products were analyzed by the MELT program [Lerman and Silverstein, 1987] in order to identify potentially high melting domains. Thirty-two regions of the gene were amplified with 10 pmoles of each primer in a 100 μl reaction mixture containing 0.5 μg of genomic DNA. Standard PCR buffer and *Taq* polymerase were used as specified by the manufacturer (Perkin-Elmer Cetus, Norwalk,

TABLE I. CSGE Analyses of COL2A1 in Affected Member (III-4) From the Original Stickler Syndrome Kindred

Region[a] containing heteroduplex species	Polymorphism detected	Mutation detected
17–20		$A^{-2}\rightarrow G$ (IVS17)
32–33	−22 exon 33 (g→a)	
34–35	$CTG^{587}\rightarrow CTT$	
38	nd[b]	

[a] Cited regions list inclusive exons and are bounded at the 5′ and 3′ ends by approximately 50 bp of intron sequence.
[b] nd, not detected.

CT). Cycling was performed in a GeneAmp PCR System 9600 thermocycler as previously described [Williams et al., 1992]. Following amplification by the PCR, 20 μl of each sample was heated to 98°C for 5 min followed by incubation at 68°C for 1 hour to generate heteroduplexes. Four microliters of the sample was mixed with an equal volume of 20% ethylene glycol (v/v)/30% formamide (wt/vol) containing 0.025% each of xylene cyanol FF and bromophenol blue. The samples were applied to a standard size sequencing gel using a 1 mm × 36 well comb. Details of the running buffer, and electrophoretic conditions have been described elsewhere [Ganguly et al., 1993]. However, the gel composition has been modified as follows: a stock solution of 39 g of acrylamide and 0.4 g of bis-acrolylpiperazine (BAP; Fluka Chemical Corp., Ronkonkoma, NY) is dissolved in 100 ml of sterile, distilled water and used to prepare a 10% polyacrylamide gel containing 10% (vol/vol) ethylene glycol (Sigma Chemical Co., St. Louis, MO), and 15% (wt/vol) formamide (GIBCO/BRL, Gaithersburg, MD). It should also be noted that the composition of 1× TTE is 89 mM Tris/29 mM taurine buffer/0.5 mM EDTA, pH 9.0 (United States Biochemical Co., Cleveland, OH).

Manual Dideoxynucleotide Sequencing of Genomic DNA

For manual dideoxynucleotide sequencing, genomic DNA was amplified by the PCR [Saiki et al., 1985] with an asymmetric ratio of primers to generate single-stranded templates [Williams et al., 1992]. Prior to sequencing, PCR products were purified by precipitation with polyethylene glycol [Kusukawa et al., 1990] and the precipitate was resuspended in deionized water.

Manual dideoxynucleotide sequencing was performed on the single-stranded templates using Sequenase Version 2.0 (United States Biochemical Co.), the nucleotide analog 7-deaza-dGTP, and ^{35}S-dATP as the labeled nucleotide [Sanger et al., 1977; Barnes et al., 1983]. Sequencing reactions were electrophoretically analyzed on a 6% polyacrylamide/urea gel. The gel was then fixed, dried, and exposed to X-ray film overnight.

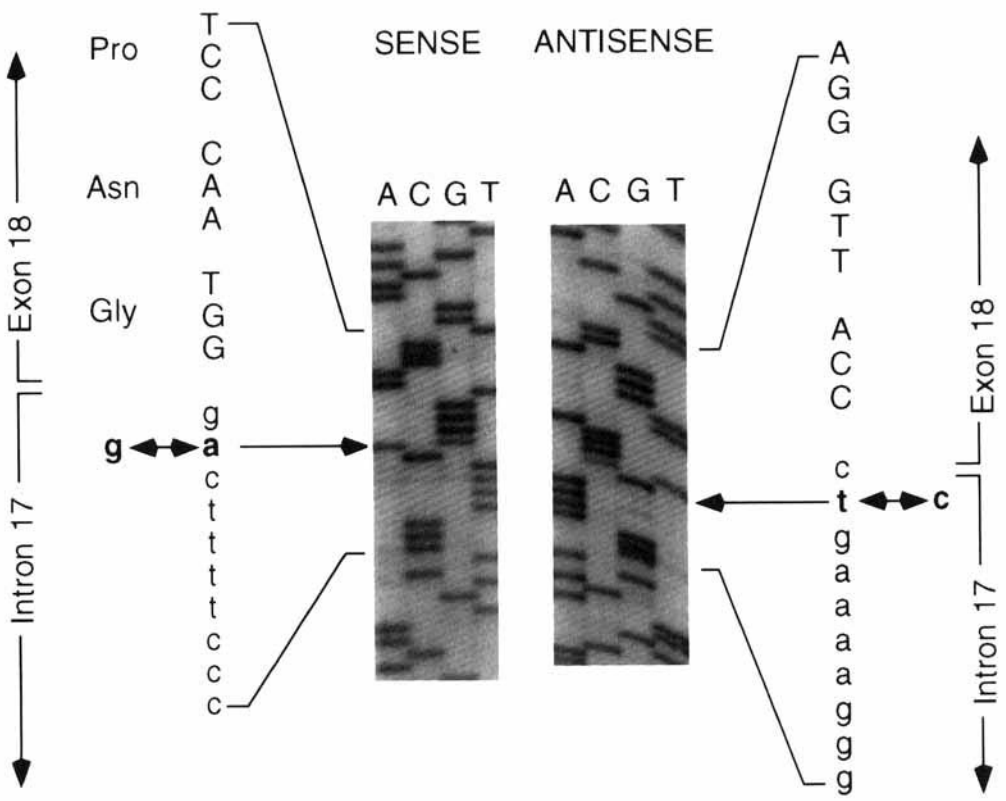

Fig. 2. Sequence analysis of a genomic DNA PCR product depicting the IVS17 splice site mutation. Manual dideoxynucleotide sequence analysis from an affected member (III-4). The region of base substitution at the IVS17 splice acceptor site is indicated by an arrow. The PCR primers for exons 17–19 are: 5′ (sense): agtgctgtcgttgcagctgg; 3′ (antisense): gactccagagatgtcagtggaac. The sequencing primers are: 5′ (sense); tggatctggatcctggag; 3′ (antisense): aggcaggactgggctctc. Direct sequencing of the genomic DNA PCR product allows visualization of both normal and mutant alleles. Sense and antisense sequencing results are shown. The eukaryotic consensus splice acceptor site sequence is: $(Py)_nNPyAG^{-1}\ G^{+1}$. The normal splice acceptor site sequence for IVS17 is: ttttcag^{-1} G^{+1}GT . . . The mutant splice acceptor site for IVS17 is: ttttcgg^{-1} G^{+1}GT . . . (Note: lowercase letters are used to denote intron sequences; upper case letters denote exon sequences. The base substitution in the −2 position of the 3′ acceptor splice site is printed in boldface.)

Verification of sequence variations was performed by restriction site analysis as previously described [Williams et al., 1992, 1995].

Reverse Transcriptase-PCR Amplification of Poly A+ RNA

Polyadenylated messenger RNA was isolated from EBV-transformed and cultured lymphoblasts by oligo dT membrane capture chromatography using a commercially available kit (United States Biochemical Co.). The membrane-captured RNA was then used for reverse transcriptase-PCR (RT-PCR) [Chan and Cole, 1991]. The reverse transcriptase reaction was primed with 50 pmoles of the antisense primer positioned at the 3′ end of exon 19. PCR amplification was completed by using sense and antisense primers (50 pmoles of each) located in exons 8 and 19, followed by a nested amplification with sense and antisense primers (20 pmoles of each) located in exons 10 and 19. Primer sequences are noted in the legend to Figure 3. PCR amplifications were performed in a GeneAmp PCR System 9600 thermocycler using the following conditions: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec for 35 cycles.

Automated Cycle Sequencing of RT-PCR Products

The RT-PCR reaction products were cloned (TA Cloning System, Invitrogen Corp., San Diego, CA) and subjected to automated cycle sequencing using the sense and antisense nested PCR primers to prime the sequencing reactions. Automated cycle sequencing was performed using dideoxy terminator reaction chemistry for sequence analysis in the Model 373A DNA sequencing system (Applied Biosystems Inc.).

RESULTS

Figure 1 depicts the partial pedigree of the kindred analyzed in this study. CSGE [Ganguly et al., 1993] was used to screen for mutations in all 54 exons of the COL2A1 gene in an affected member from the kindred (III-4). Table I summarizes the results of these analyses. Four regions exhibited heteroduplex species; the exons within these regions, and 50 bp upstream and downstream of each exon boundary, were subjected to manual dideoxynucleotide and automated cycle sequencing analysis. Two of the four species were found to result from neutral polymorphisms. The sequence variation giving rise to one heteroduplex species was not identified; it probably resided well into the intron beyond the region that was subjected to sequence analysis. However, when direct sequencing of PCR-amplified genomic DNA from an affected member was undertaken for the exon 17–20 area of the gene, a base substitution was identified in the A^{-2} position of the 3′ acceptor splice site of IVS17 (Table I and Fig. 2). The mutation was confirmed by antisense sequencing of the gene at this position (Fig. 2) and by restriction enzyme digestion (data not shown) using a PCR-introduced HpaII restriction site to distinguish the normal and mutant alleles. DNA from several affected and unaffected family members was subsequently PCR-amplified and analyzed for the base substitution; results of these analyses confirmed that the mutation was present in affected members only (data not shown). The mutation was absent in DNA from 114 normal, unrelated control subjects.

To determine if the mutation affected the splicing of COL2A1 mRNA transcripts, cDNA from the proband was generated by RT-PCR of lymphoblast poly A+ RNA [Chan and Cole, 1991]. The normal allele gave rise to a transcript that was of the expected size and sequence (Fig. 3A). However, the mutant allele gave rise to a single transcript that was slightly shorter as a result of the inability of the splice donor in intron 17 to utilize the mutant acceptor splice site. Sequence analysis showed that a cryptic splice site in exon 18 was utilized as the new splice acceptor in the mutant allele, resulting in the deletion of 16 bp from the start of exon 18 (Fig. 3B). Despite this frameshift deletion, the nucleotide sequence of exon 19 was conserved in the transcript from the mutant allele, suggesting that RNA from this allele was processed. This outcome would predict, therefore, the generation of a premature termination codon 645 bp downstream from the inappropriate splice site.

DISCUSSION

The type II collagenopathies include several pathogenetically related syndromes whose phenotypic presentations span a wide range of clinical extremes. They are inherited in an autosomal dominant manner. Mutations in the type II collagen molecule presumably result in either a dominant negative effect or the effect of haploinsufficiency that is reflected in compromise of cartilage integrity [for reviews see Spranger et al., 1994 and Vikkula et al., 1994].

These findings are the first demonstration of a splice site mutation in the classical, ocular form of Stickler syndrome. Interestingly, the outcome of the mutation, that is the generation of a premature termination codon from the mutant allele transcript, is entirely consistent with most other reported COL2A1 mutations for this syndrome. The results, therefore, provide a satisfying historical context in which to view COL2A1 mutations in Stickler syndrome. They also support the hypothesis that, although they both impact on ocular tissues and are caused by mutations in the same gene, there exists a molecular distinction between mutations in the COL2A1 gene that cause ocular and skeletal symptoms (characteristic of Stickler syndrome) versus those that cause hyaloideoretinal degeneration but without skeletal changes, as has been reported for a case of Wagner syndrome in which a glycine to asparagine substitution was observed [Korkko et al., 1993].

Presumably, the mild to moderate phenotype which characterizes Stickler syndrome results from reduced COL2A1 gene product rather than from the deleterious effects of "protein suicide" [Prockop and Kivirikko, 1984]. However, it is becoming clear that the Stickler syndrome phenotype may not arise exclusively as the result of a premature termination of COL2A1 tran-

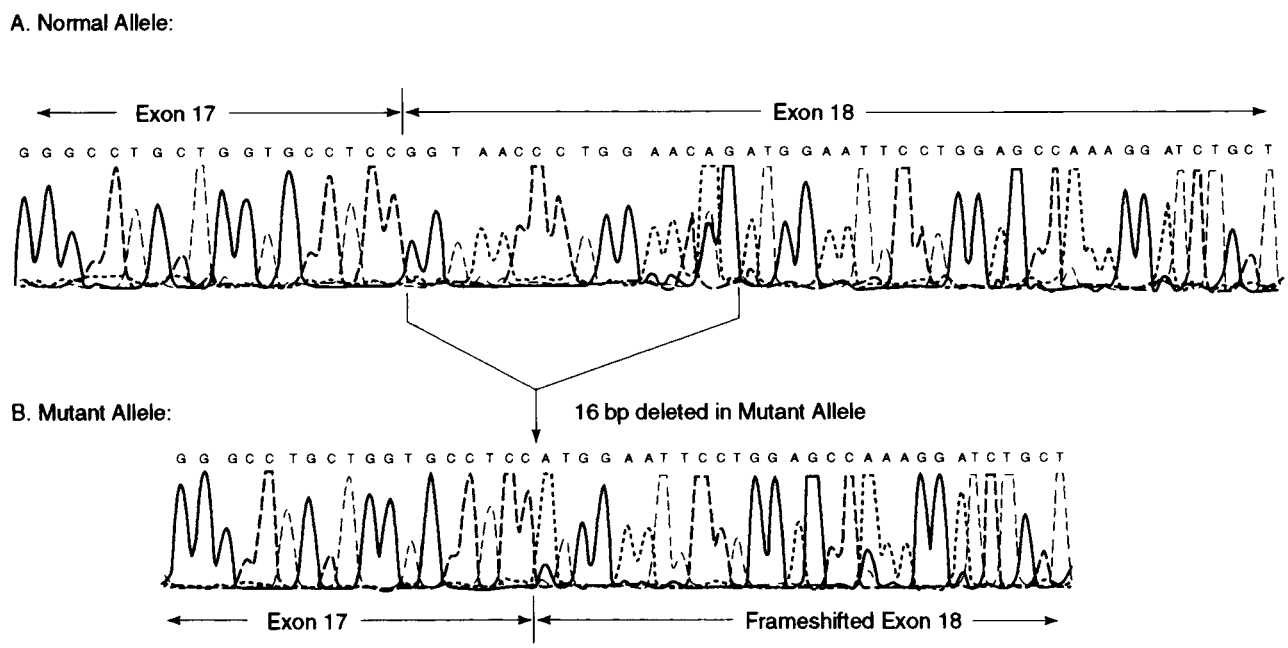

Fig. 3. Automated cycle sequencing of RT-PCR products from an affected member (III-4). The primer for the RT reaction was: 3′ (antisense); GAGGACCAGTTGCACCTT. The PCR primers were: 5′ (sense): AAGGATTTCAAGGCAATC; 3′ (antisense): GAGGACCAGTTGCACCTT. The nested PCR primers were: 5′ (sense); GCTGGAAAACCTGGAAAAG; 3′ (antisense): GAAGCCAGGAGCACCAGCAATGC. Sequencing was accomplished with both sense and antisense nested PCR primers; only the sense direction is shown. **A:** Sequencing of the RT-PCR product from the normal allele. The normal sequence was obtained. Sequence of the deleted area in the mutant allele is indicated in brackets. **B:** Sequencing of the RT-PCR product from the mutant allele. (Normal type II collagen cDNA sequences are from Baldwin et al. [1989]; Genbank Accession Number X16711).

scription/translation. One other case of Stickler dysplasia has been reported in which the patient was mosaic for a 28 bp deletion spanning the 3′ exon/intron boundary of exon 12. The authors concluded that the effect of this splice site deletion was the skipping of exon 12 and the subsequent deletion of 18 amino acids from the mature type II procollagen chain [Winterpacht et al., 1993]. While it might be postulated that such a mutation could result in protein suicide, the patient was only mildly affected, presumably as a consequence of her mosaic status. However, her daughter who was affected with the same mutation but was not mosaic, manifested the more severe Kniest dysplasia phenotype.

It should also be noted that over half of all families with Stickler syndrome are not linked to COL2A1 [Francomano et al., 1988; Fryer et al., 1990]. A nonocular form of Stickler syndrome has been linked to the chromosomal interval near COL11A2 [Brunner et al., 1994]. In a recent report, the clinical phenotype of a kindred with this autosomal dominant syndrome was found to be caused by a 5′ splice site mutation in the COL11A2 gene leading to in-frame exon skipping [Vikkula et al., 1995]. Therefore, it is not unreasonable to expect that other extracellular matrix genes which are expressed in skeletal and ocular tissues may be involved in the development of the Stickler syndrome phenotype.

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